

An evaluation of *in vitro* screening techniques for determining tolerance of avocado rootstocks to *Phytophthora cinnamomi*

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ABSTRACT

Different in vitro screening techniques were used to quantitatively compare four avocado rootstocks for susceptibility to Phytophthora cinnamomi. Attraction of P cinnamomi zoospores by exudates from excised avocado roots, diffusing through a dialysis membrane, accurately reflected tolerance or susceptibility and eliminated the need for laborious processing of roots for microscopy. Similarly, lesion development on detached roots and leaves, after inoculation with the pathogen, correlated well with field performance of the various rootstocks, yet was simple to perform.

UITTREKSEL

Verskillende tegnieke vir in vitro toetsing is gebruik om vier avokadoonderstamseleksies te vergelyk vir vatbaarheid vir Phytophthora cinnamomi. Aantrekking van P cinnamomi soóspore deur uitskeiding van afgesnyde avokadowortels oor 'n dialisemembraan, het bekende bestandheid of vatbaarheid akkuraat weerspieël en het omslagtige prosessering van wortels vir mikroskopie uitgeskakel. Insgelyks het letselontwikkeling op afgesnyde wortels en blare, na inokulasie met die patogeen, goed gekorreleer met veldbestandheid van die verskillende onderstamme en was verder maklik om uit te voer.

INTRODUCTION

The omnipresence and severity of avocado root rot in South Africa justifies a search for tolerant rootstocks (Ben-Ya'Acov, 1985). Avocado trees exist in these countries which show little or no apparent root rot development in soils infested with *P cinnamomi*. The potential of these trees prompted evaluation of and comparison to various *in vitro* techniques, and investigation into the modification of these procedures, as well as new methods for screening tolerance of avocado roots to *P cinnamomi*. Results of these investigations are presented in this paper.

MATERIALS AND METHODS

Plant material

Roots obtained from 10 to 12-month-old susceptible *P americana* cv Edranol seedlings (Snyman, Snyman & Kotzé, 1984), and vegetatively propagated (Frolich & Platt, 1971) moderately tolerant seedlings of *P americana* selections Duke 7 and G6 (Coffey, 1987), as well as *P schiedeana* Nees selection G755, tolerant according to Coffey (1987), were used in all experiments.

Pathogen isolate and zoospore production

P cinnamomi (PREM 49103, unknown mating tape) isolated from avocado roots collected in the Tzaneen area, was grown at 25°C on pea agar (Chen & Zentmyer, 1970). Sporangia production was stimulated by using the method of Gisi, Zentmyer & Klure (1980). Zoospore release was induced by washing the mycelial mats thrice in sterile distilled water and chilling for 60 minutes at 10°C. A concentration of 10^5 - 10^6 ml⁻¹ zoospores was obtained in this manner.

Electrolyte leakage

Freshly cut root tips (15 mm long) of the various rootstocks were tested for tolerance to *P cinnamomi* with the detached root inoculation technique of Zilberstein & Pinkas (1987). Electrolyte leakage from inoculated roots was measured with a conductivity meter. The correlation between electrolyte leakage (µs/cm) and susceptibility was determined.

Zoospore attraction

Mehrotra (1970) previously described a technique to demonstrate attraction of zoospores of *Phytophthora drechsleri* Tucker and *P megasperma* var *solae* (Drechsler) Hildebrand by exudates from safflower and soybean roots. Based on this principle, a method was developed to measure zoospore attraction to roots of the different avocado rootstocks. One hundred microlitre of the *P cinnamomi* zoospore suspension, containing $3,9 \times 10^5$ ml⁻¹ motile zoospores, were introduced into sterile 2 ml Eppendorf tubes. The tubes were filled completely with sterile distilled water and each was sealed with a dialysis membrane (Spectrapor). A freshly cut root tip ca 15mm long was placed on each membrane (18 per rootstock), with the cut end protruding over the edge of the membrane (Figure 1). This prevented contact between the cut ends and the membrane, thus precluding attraction of zoospores to the wounds. The tubes with roots were incubated at 25°C in a dark, moist chamber. After 1,5 and 4h respectively, nine membranes were removed and stained with 0,5 per cent cotton blue in lactophenol (Figure 2). Zoospore encystment and germination were examined microscopically. To evaluate the zoospore attraction technique further, root tips (six per rootstock) were individually placed in test tubes (1,5 X 15cm), each containing 2 ml sterile distilled water. Ten microlitre of the *P cinnamomi* zoospore suspension containing $3,9 \times 10^5$ ml⁻¹ motile zoospores were added to each tube. The tubes were incubated in the dark for

three h, whereafter root tips were fixed in 6 per cent glutaraldehyde buffered with 0,1 M sodium cacodylate (pH 7,35) and rinsed in three changes of 0,1 M sodium cacodylate buffer. An ethanol series (50, 70, 90 and 100 per cent) was used for dehydration. Critical point drying was accomplished in a Hitachi HCP-2 critical point drier (Hitachi Koki Co Ltd, Tokyo, Japan). Gold coating was done with an Eiko B3 ion coater (Eiko Engineering Co Ltd, Japan). Root segments were viewed under a Hitachi S450 scanning electron microscope (SEM) (Hitachi Ltd, Tokyo, Japan) at 15 kV zoospores encystment and germination of *P cinnamomi* was recorded.

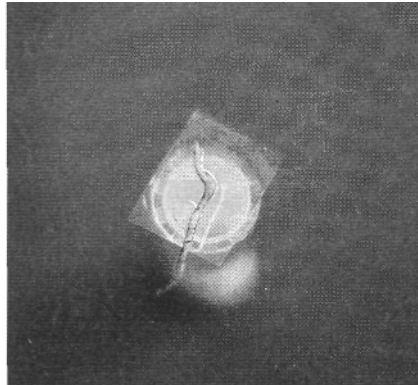


Fig 1 The dialysis membrane technique with the avocado root in position on the membrane.

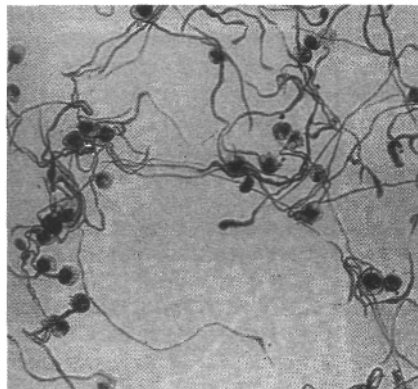


Fig 2 Encystment and germination of *P cinnamomi* zoospores on the membrane.

Tissue colonisation

Roots

Estimating tolerance of avocado root-stocks to *P cinnamomi* by determining colonisation of excised root tips (Kellam & Coffey, 1985; Dolan & Coffey, 1986), is often complicated by organisms contamination of the plating medium (unpublished data). In the present

investigation, this problem was circumvented by preventing root tip contact with the medium. Water agar (1 per cent) was poured into 85 mm diameter petri dishes at a rate of 15 ml per dish. Before the agar solidified, two sterile glass rods (7mm diameter, 60 mm long) were placed parallel to each other into the agar, ca 15mm apart. Root tips of the various root-stocks, ca 40 mm long and with known diameter, were placed perpendicularly on the glass rods in the petri dishes (15 root tips per plate, four plates per rootstock). Each root tip was inoculated at the region of elongation with 10 µl of the *P cinnamomi* zoospore suspension, containing $4,1 \times 10^5 \text{ml}^{-1}$ motile zoospores, and the plates were incubated in the dark at 25° C. After 24 h and 48 h respectively, 30 root tips of each rootstock were removed and the length of the lesions recorded. Seven root tips of each rootstock, collected after 48 h, were surface disinfested for 5 s in 70 per cent ethanol, cut aseptically into 3mm segments and the segments arranged sequentially on potato dextrose agar plates according to distance from the root tip. After 3 d, incubation of the plates at 25°C, the segments from which *P cinnamomi* developed were recorded. A further seven root tips of each rootstock, collected after 48 h, were macerated separately in 5 ml sterile distilled water for 10 s with an Ultra Turrax. From each suspension, 200 µl was plated on PARPH medium (Kannwischer & Mitchell, 1978), and the plates were incubated at 25°C. After 3d, the number of *P cinnamomi* colonies that developed were counted and expressed as colony forming units (cfu) per 0,2l root tissue.

To determine whether roots differing in diameter differed in susceptibility to *P cinnamomi*, lesion length in 10 visually thin and 10 visually thicker root tips of each of the rootstocks was compared after 48 h. The mean diameter of the former root tips was 1,1 mm and that of the latter 2,0 mm. These diameters differed significantly at a 1 per cent probability level.

Leaves

Ten young (ca two weeks old) and ten older (more than six weeks old) leaves of each rootstock were removed, wounded centrally with a sterile needle, and each leaf point inoculated with 10 µl zoospores suspension containing $1,7 \times 10^5 \text{ml}^{-1}$ motile zoospores. Lesion development was recorded after 3d incubation at 25°C in a moist chamber (Figure 3).

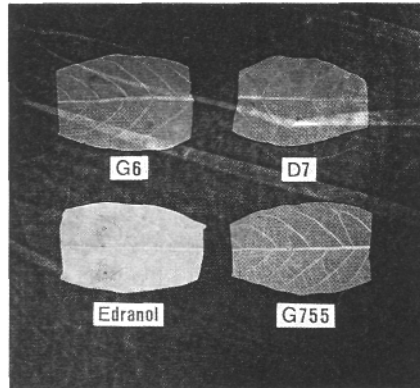


Fig 3 Lesion development on an excised avocado leaf after inoculation with *P. cinnamomi* zoospores.

RESULTS

Electrolyte leakage

Electrical conductivity (EC) values of bathing solutions incubated with inoculated root segments from Edranol, Duke 7 and G6, did not differ significantly 48 h after inoculation, but were significantly greater than that of the tolerant G755 (Table 1). After 72 h and 96 h the sequence changed and EC did not correlate with the reported tolerance of the various rootstocks.

TABLE 1 Electrical conductivity of bathing solutions incubated with avocado root segments from four avocado rootstocks inoculated with *P. cinnamomi*

Root-stock	Electrical conductivity ($\mu\text{S}/\text{cm}$)*		
	Incubation time (h)		
	48	72	96
Edranol	121 a	85c	30c
Duke 7	99a	123b	117b
G6	120a	178a	128b
G755	66b	128b	173a

*Each value is the mean of six replicates. Values for uninoculated root segments were subtracted from values for inoculated root segments. In columns, values not followed by the same letter differ significantly according to Duncan's multiple range test ($P=0,05$).

Zoospore attraction

The three tolerant rootstocks (Duke 7, G6 and G755) attracted significantly less zoospores than the susceptible Edranol, when evaluated according to the DM-technique

(Table 2). These results correlated well with the root-stocks observed under SEM (Table 3).

TABLE 2 Zoospore encystment of *P cinnamomi* on root tips of four avocado rootstocks determined by means of the dialysis membrane technique

Rootstock	Number of cysts per root tip*	
	Incubation time (h)	
	1,5	4
Edranol	82a	37a
Duke 7	17a	6b
G6	6b	8b
G755	2b	3b

*Each value is the mean of nine replicates. In columns, values not followed by the same letter differ significantly according to Duncan's multiple range test (P = 0,05).

TABLE 3 Zoospore encystment and germination of *P cinnamomi* on root tips of four avocado rootstocks observed under SEM

Rootstock	Number of cysts per root*	Cyst germination (%)*
Edranol	123a	78a
Duke 7	62b	58b
G6	57b	63b
G755	26b	49b

*Each value is the mean of six replicates. In columns, values not followed by the same letter differ significantly according to Duncan's multiple range test (P = 0,05).

Tissue colonisation

Roots

Based on mean lesion length, G755 exhibited the highest degree of resistance to colonisation by *P cinnamomi*; Duke 7 and G6 less, and Edranol the least (Figure 4; Table 4). Significant differences in linear root colonisation of roots from the point of inoculation were evident between G755 and the other three rootstocks. G6 and G755 yielded significantly less propagules per 0,2l root tissue than Edranol (Table 5). Lesion development on thin root tips of each cultivar did not differ significantly from lesion development on thick root tips.

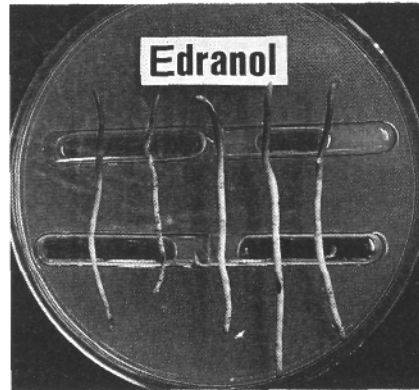


Fig 4 Lesion development on excised Edranol roots, 48 h after inoculation with *P cinnamomi*.

TABLE 4 Lesion development on excised root tips of four avocado rootstocks inoculated with *P cinnamomi*

Rootstock	Length of lesion (mm)*	
	Incubation time (h)	
	25	48
Edranol	8,4a	15,8a
Duke 7	4,6b	10,0b
G6	5,6b	12,0b
G755	1,8c	5,6c

*Each value is the mean of 30 replicates. In columns, values not followed by the same letter differ significantly according to Duncan's multiple range test (P=0,05).

TABLE 5 Linear colonisation of root tips of four avocado rootstocks by *P cinnamomi* and the incidence of the pathogen in root tissue after artificial inoculation

Rootstock	Linear colonisation of feeder roots (mm*)	Number of <i>P cinnamomi</i> cfu recovered per 0,2 ml root tissue**
Edranol	26,8a	2,9a
Duke 7	24,6a	2,3ab
G6	24,4a	1,3bc
G755	14,5b	0,9c

*Roots were inoculated at the tip with zoospores. After 2d roots were cut in 3 mm segments and the segments plated on PDA. Each value is the mean of seven replicates.

**Roots were macerated and plated on PARPH medium 2d after inoculation. The number of colonies of *P cinnamomi* per plate were counted and expressed as cfu per 0,2 ml root tissue. Each value is the mean of seven replicates.

Values followed by the same letter do not differ significantly according to Duncan's multiple range test (P = 0,05).

Leaves

Lesions developed on young leaves of all the rootstocks after inoculation with Duke 7, G6 and G755, which showed a significantly higher degree of resistance than Edranol. On older leaves, lesion development was restricted (Table 6).

TABLE 6 Lesion development on excised leaves of four avocado rootstocks after inoculation with *P cinnamomi*

Rootstock	Lesion diameter after 3d (mm)*	
	Young leaves	Old leaves
Edranol	12,3a	0,3a
Duke 7	2,4b	0,1a
G6	3,0b	0,2a
G755	0,9b	0,2a

*Each value is the mean of ten replicates. In columns, values not followed by the same letter differ significantly according to Duncan's multiple range test (P=0,05).

DISCUSSION

In this investigation, new and existing techniques for the *in vitro* evaluation of tolerance to *P cinnamomi* in avocado rootstocks were compared quantitatively. In addition, shortcomings identified in some of the techniques were rectified to make them more suitable for screening purposes.

Although tolerance and susceptibility could be distinguished by all the methods, measuring lesion development on and linear colonisation of excised root tips, after inoculation with the pathogen proved to be the most effective in terms of reliability and simplicity. The correlation between lesion development and linear colonisation is in accordance with previous reports on other crops (Umaerus & Lihnell, 1976; Byrt & Holland, 1978; Blaker & Hewitt, 1987) and illustrates the importance of these two parameters as components in the tolerance of avocado selections to *P. cinnamomi*. Expression of tolerance was not affected by root thickness. Dolan & Coffey (1986) reported similar observations for Duke 7 and G6, but found that lesion development on thin roots of G755 was significantly more severe than on thick roots. This could be ascribed to the fact that the thick roots used by Dolan & Coffey (1986) had a mean diameter of 3mm compared to 2mm in the present investigation. However, since the majority of avocado roots in the field are less than 2mm in diameter (Salazar-Garcia & Cortes-Flores, 1986), the reportedly lower tolerance of thicker roots of G755 should be of little significance.

When using detached roots for evaluating tolerance in field trees, as described by Kellam & Coffey (1985) and Dolan & Coffey (1986), the excised material cannot be disinfested superficially. The roots therefore harbour contaminating microorganisms, which can complicate observations and eventually affect the reliability of the results. In the present study, this problem was overcome by suspension of the excised root tips on glass rods, thus preventing contact with the medium, while providing an atmosphere with sufficient moisture to maintain cell viability.

Electrolyte leakage, although a relatively simple and rapid technique for indicating tolerance (Zilberstein & Pinkas, 1987), required a substantial number of roots, necessitated time-consuming calculations and gave inconsistent results (unpublished data). Similarly, determining methods described so far for zoospore attraction of *P. cinnamomi* by avocado roots (Zentmyer, 1961; Aveling, 1988), all involve examination of the roots by light or scanning electron microscopy and therefore laborious processing of the roots. Modification and application of the dialysis membrane technique of Mehrotra (1970) to the screening of excised avocado roots required considerably less time and effort, yet results which satisfactorily reflected known tolerance or susceptibility were obtained. In addition, the membranes allowed diffusion of exúdales only, thus eliminating contaminating microorganisms.

Inoculation of young avocado leaves with *P. cinnamomi* and measuring lesion development holds promise as a screening technique for pathogen tolerance. It should be particularly useful for screening ungrafted field trees, as well as for evaluating vegetatively propagated seedlings without disturbing their roots. Brune & Van Lelyveld (1982) previously found an association between phenolic compounds in mature leaves of different avocado selections, and tolerance of these selections to *P. cinnamomi*. However, in the present investigation, differences in lesion development between selections were evident in young leaves only. This implies that should phenolic compounds be involved in the expression of tolerance, differences in the concentration of these compounds in leaves of tolerant and susceptible rootstocks will be the greatest in young leaves.

In summary, the dialysis membrane techniques, as well as lesion development on

excised roots and leaves, appear to be the best suited for *in vitro* screening of potential root rot resistance in candidate avocado selections. Rootstocks selected in this way will obviously have to be evaluated in the greenhouse and field before being recommended for commercial use.

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REFERENCES

- AVELING, TAS, 1988. Resistance/susceptibility phenomena in the avocado-*Phytophthora cinnamomi* interaction. MSc Thesis, University of Natal, Pietermaritzburg.
- BEN-YA'ACOV, A 1985. Selection of avocado rootstocks. *SA Avocado Growers' Assoc Yrb*, 81, 21 - 23.
- BLAKER, N S & HEWITT, J D 1987. A comparison of resistance to *Phytophthora parasitica* in tomato. *Phytopathology*, 77, 1113 - 1116.
- BRUNE, W & VAN LELYVELD, L J, 1982. Biochemical comparison of leaves of five avocado (*Persea Americana* Mill) cultivars and its possible association with susceptibility to *Phytophthora cinnamomi* root rot. *Phytopathological Zeitschrift*, 104, 243 - 254.
- BYRT, PN & HOLLAND, A A, 1978. Infection of axenic Eucalyptus seedlings with *Phytophthora cinnamomi* zoospores. *Australian Journal of Botany*, 26, 169 - 176.
- CHEN, DW & ZENTMYER, G A, 1970. Production of sporangia by *Phytophthora cinnamomi* in axenic culture. *Mycologia*, 62, 397 - 402.
- COFFEY, M D, 1987. A look at current avocado rootstocks. *California Grower*, 11, 15 - 17.
- DOLAN, TE & COFFEY, M D, 1986. Laboratory screening technique for assessing resistance of four avocado rootstocks to *Phytophthora cinnamomi*. *Plant Disease*, 70, 115 - 118.
- FROLICH, EF & PLATT, R G, 1971. Use of the etiolation technique in rooting avocado cuttings. *California Avocado Soc Yrb*, 56, 97 - 109.
- GISI, U, ZENTMYER, GA & KLURE, LJ, 1980. Production of sporangia by *Phytophthora cinnamomi* and *P palmivora* in soils at different matric potentials. *Phytopathology*, 70, 301 - 306.
- KANNWISCHER, M E & MITCHELL, J D, 1978. The influence of a fungicide on the epidemiology of black shank of tobacco. *Phytopathology*, 68, 1760 - 1765.
- KELLAM, M K & COFFEY, M D, 1985. Quantitative comparison of the resistance to *Phytophthora* root rot in three avocado rootstocks. *Phytopathology*, 75, 230 - 234.
- MEHROTRA, R S, 1970. Techniques for demonstrating accumulation of zoospores of *Phytophthora* species in soil. *Canadian Journal of Botany*, 48, 879 - 882.
- SALAZAR-GARCIA, S & CORTES-FLORÉS, J I, 1986. Root distribution of mature avocado trees growing in soils of different texture. *California Avocado Soc Yrb*, 70,

165 - 173.

- SNYMAN, A J, SNYMAN, C P & KOTZÉ, J M, 1984. Pathogenicity of avocado root rot fungi to Edranol seedlings and Duke 7 rooted cuttings. *SA Avocado Growers' Assoc Yrb*, 7, 80 - 81.
- UMAERUS, V & LIHNELL, D, 1976. A laboratory method for measuring the degree of attack by *Phytophthora infestans*. *Potato Research*, 19, 91 - 107.
- ZENTMYER, G A, 1961. Chemotaxis of zoospores for root exúdales. *Science*, 133, 1595 - 1596.
- ZENTMYER, G A, 1980. *Phytophthora cinnamomi* and the diseases it causes. Phytopathological Monograph 10. American Phytopathological Society, St Paul, MN.
- ZILBERSTEIN, M & PINKAS, Y, 1987. Detached root inoculation a new method to evaluate resistance to *Phytophthora* root rot in avocado trees. *Phytopathology*, 77, 841 - 844.